Discovery of prognostic markers in laryngeal cancer treated with radiotherapy

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Chapter 7

Summary and general discussion

SUMMARY

Laryngeal squamous cell carcinoma (LSCC) is the second most common head neck cancer with approximately 211,000 new cases worldwide and 700 cases in the Netherlands annually[1,2]. Choice of treatment and prognosis of LSCC is nowadays mostly based on well-known clinicopathological factors such as subsite and tumor stage[3-5]. In general, LSCC is most often treated with radiotherapy as a single modality with exceptions of very early stage (T_{1a}) and (locoregionally) advanced stages (T_4, N_{2-3}). T_{1a} tumors are often treated with transoral laser surgery. In locoregionally and locally advanced stages chemoradiation or total laryngectomy (if no functional larynx can be expected after treatment) are treatments of choice, respectively. Because of the high local recurrence rate after primary radiotherapy (approximately 25%), new prognostic markers are needed to predict treatment response.

The main goal of this thesis was to investigate several potential biomarkers in the pre-treatment tumor biopsies that might be prognostic for the treatment response after definitive radiotherapy in patients with LSCC and to improve treatment selection which is currently solely based on clinical characteristics. We also evaluated the applicability of a hypoxia PET tracer with immunohistochemical hypoxia markers. We constructed a large database with clinicopathological and follow-up data of over 1286 patients with LSCC diagnosed at the department of Otorhinolaryngology / Head & Neck Surgery, treated in collaboration with the department of Radiation Oncology at the University Medical Center Groningen (UMCG) and of which pre-treatment tumor biopsies were available in the archives of the department of Pathology of the UMCG. From this database it was possible to select a well-defined homogenous group of patients with stage T_1-T_2 histologically confirmed LSCC treated with definitive radiotherapy.

Tumor hypoxia is frequently seen in head and neck squamous cell carcinoma (HNSCC) and is related to radioresistance and consequently worse locoregional tumor control after radiotherapy[6-9]. Several techniques to measure tumor hypoxia are reported, like invasive measurement using Eppendorf needle electrodes, exogenous markers (pimonidazole), endogenous hypoxia related tumor markers (HIF1α, GLUT-1, CA-IX) and biological hypoxic tracer imaging, for instance with ^{18}F-MISO or ^{18}F-FAZA-PET scanning. However, the optimal way to measure hypoxia and tissue distribution is still not clear. The use of biological hypoxic tracer imaging with PET is promising since it is not invasive, it can visualize the

whole tumor and might identify intratumoral hypoxia heterogeneity. Therefore, it can be helpful in planning and monitoring treatment for instance hypoxia-based radiotherapy schedules[10,11]. Most hypoxia imaging studies on head and cancer have been performed using ^{18}F-labeled fluoromisonidazole (^{18}F-FMISO). Later, another hypoxia PET tracer, ^{18}F-fluoroazomycinarabinoside (^{18}F-FAZA) which is a chemically related molecule to ^{18}F-FMISO was also reported. In chapter 2, we summarized the literature including both animal and human ^{18}F-FAZA-PET studies and specifically discussed how individualized treatment could be applied in patients with hypoxic head and neck tumors. Our analysis revealed that ^{18}F-FAZA-PET imaging is feasible to detect tumor hypoxia and have superior biokinetics compared with ^{18}F-FMISO. Therefore, ^{18}F-FAZA is a promising PET radiopharmaceutical for visualization of tumor hypoxia, although clinical studies must still confirm the clinically applicable role of ^{18}F-FAZA-PET scanning in head and neck oncology.

Because ^{18}F-FAZA is a promising hypoxia radiopharmaceutical agent, in chapter 3, we aimed to determine the accuracy of ^{18}F-FAZA-PET/CT scan by comparing hypoxic regions detected by ^{18}F-FAZA-PET/CT scan with expression levels of various immunohistochemical hypoxia tumor markers in LSCC patients. For this purpose, in 11 patients ^{18}F-FAZA-PET scan was performed before total laryngectomy and hypoxic regions were determined. These regions were spatially related with immunohistochemical expression of exogenous (pimonidazole) and endogenous (HIF1α, CA-IX and GLUT-1) hypoxia markers of the laryngectomy specimen. Inter- and intratumoral heterogeneity of tumor hypoxia was observed on ^{18}F-FAZA-PET scan. Nine of the 11 tumors were found hypoxic with ^{18}F-FAZA-PET imaging. Hypoxia could also be detected with pimonidazole, HIF1α, CA-IX and GLUT-1 expression in some tumors. However, no clear association was observed between ^{18}F-FAZA uptake and hypoxia markers. This study showed no relation between the hypoxic regions detected by ^{18}F-FAZA-PET scanning and by the well-reported endogenous immunohistochemical hypoxic markers in laryngeal cancer. However, the low number of cases does not allow us to draw firm conclusions. Further studies on larger sample size are needed.

The activation of PI3K/AKT antiapoptotic and proliferation pathway may contribute to tumorigenesis and hence a worse prognosis in many cancer types, including LSCC[12-14]. The PI3K/AKT pathway can be triggered by activation of the epidermal growth factor receptor (EGFR). Another mechanism for PI3K/AKT pathway activation is the loss of phosphatase and tensin homolog deleted on chromosome 10 (PTEN), a tumor suppressor gene which opposes PI3K/AKT activation. High

expression of EGFR and PTEN are found to have an association with response to radiotherapy in HNSCC, resulting in decreased local control[12-19]. Despite EGFR expression in >95% of HNSCC's[20,21], only 10-16% of patients benefit from therapy targeting the EGFR molecule[20,21] and thus implying that EGFR expression levels are not predictive for targeted anti-EGFR treatment response. In chapter 4, the prognostic role of immunohistochemical expression of EGFR and PTEN on local control in patients with early stage supraglottic LSCC treated with radiotherapy was investigated. Immunohistochemical staining for EGFR and PTEN was performed on pre-treatment biopsies of a selected well-defined homogeneous group of 52 patients from our database with T_1-T_2 supraglottic LSCC treated with radiotherapy between 1990 and 2008. Cox regression analysis showed a significant association between PTEN expression and local control, and between lymph node status and local control. Both were independent prognostic factors in a multivariate analysis. However, there was no significant association between EGFR expression and local control. The worse local control found in cases with high PTEN expression suggests the importance of PI3K/AKT independent functions of PTEN, such as DNA-damage repair in radioresponse. Therefore, PTEN status could have an additive value in determining the prognosis of early stage supraglottic LSCC and might be used to select patients for therapies other than primary radiotherapy alone.

In chapter 5, the prognostic role of the Ataxia Telangiectasia Mutated (ATM) and substrates genes checkpoint kinase 2 (Chk2) and p53 are investigated as prognostic markers for radioresponse in early stage LSCC. ATM, Chk2 and p53 play a critical role in DNA damage response (DDR) which controls cell cycle checkpoints, DNA repair and apoptosis induced by ionizing radiation. High levels of phosphorylated ATM (pATM) were linked to poor locoregional disease-free survival in patients with cervical cancer treated with (chemo)radiation earlier[22]. High pChk2 expression was previously found in HNSCC but associations with clinical outcome were not investigated[22-24]. Tissue samples from 141 patients selected from our database with T_1-T_2 LSCC treated with definitive radiotherapy were immunohistochemically stained with antibodies against pATM, pChk2 and p53. Cox regression analyses were performed to examine whether a high expression level of markers was associated with a poor local control. High levels of pATM were associated with significantly poor local control. pChk2 showed a non-significant trend. p53 was not prognostic for local control. In this cohort, high levels of pATM were associated with poor local control, indicating a potential important role for the DDR pathway in predicting response to radiotherapy in LSCC. These patients might

benefit from ATM inhibition treatment, resulting in sensitization for radiotherapy. Our observations warrant further independent investigation.

Methylation is a form of epigenetic regulation. Methylation of specific genes can be used as biomarker for diagnosis and prognosis[25]. Hypermethylation leads to transcriptional repression and hypomethylation to reactivation of gene transcription. Because of its dynamic nature, methylation is a possible candidate for the dynamic gene regulation in tumor progression causing local recurrence in LSCC. The DNA methyltransferases (DNMTs) including DNMT1, DNMT3a and DNMT3b play an important role in the methylation process by adding methylgroups to CpG dinucleotides. Overexpression of DNMT's is associated with hypermethylation and oncogenic activation in a variety of tumors[25]. DNMT1 overexpression was found to correlate with aberrant DNA methylation in solid tumors, including esophageal carcinomas, resulting in poor prognosis[26]. In chapter 6, we investigated the prognostic role of immunohistochemical expression of DNMT1 on a well-defined series of 125 patients with early stage LSCC, treated with radiotherapy from our database. We found an association between high DNMT1 expression and local control. The worse local control found in cases with high DNMT1 expression suggests the importance of methylation of tumor suppressor genes, important for tumor progression and radioresponse. Our findings suggest that the DNMT1 status could have an additive value as a prognostic marker for response to radiotherapy and as a possible target with DNMT inhibitors for treatment of early stage laryngeal carcinomas.

GENERAL DISCUSSION

In this thesis several biomarkers with potential prognostic value for radioresponse in laryngeal squamous cell carcinoma (LSCC) were described. First, we evaluated one of the hypoxia PET tracers, ^{18}F-fluoroazomycinarabinoside (^{18}F-FAZA). Next, immunohistochemical expression of the protein markers EGFR, PTEN, pATM, pChk2, p53 and DNMT1 were evaluated.

^{18}F-FAZA as a hypoxia PET tracer

Hypoxia characterization of the whole tumor prior, during and after radiotherapy treatment with non-invasive techniques like PET/CT imaging or MRI would be of great relevance for radiotherapy planning[10,27,28]. Furthermore, hypoxia PET scan can also be used to select patients for an alternative additional hypoxia-targeted treatment. For example, accelerated radiotherapy combined with the hyperoxic gas carbogen and nicotinamide (ARCON) is investigated in phase III trials[29]. Another option, tirapazamine, is a drug with selective cytotoxicity for hypoxic cells. In a phase III trial patients with locally advanced head and neck cancer were randomly assigned to receive chemoradiation or chemoradiation with tirapazamine. Although in this study no advantage was found[30], in a substudy tirapazamine was found to be effective in patients with hypoxic tumors as assessed by ^{18}F-FMISO-PET[31].

Several studies on different hypoxia radiopharmaceuticals have been published. Because of the diverse study setups and analysis, it is difficult to compare studies using the same PET radiopharmaceuticals. The group of nitroimidazoles is the largest of PET radiopharmaceuticals, used for hypoxia imaging. Nitroimidazole compounds undergo reduction under hypoxic conditions and form highly reactive oxygen radicals. After binding to intracellular macromolecules, they are trapped inside the hypoxic cells. When labeled with a radioisotope, for instance ^{18}F, it can be detected by a PET scanner. Among the radiolabeled nitroimidazoles, ^{18}F-FMISO is the most frequently studied. Conflicting results were found when evaluating this tracer with the exogenous hypoxia marker pimonidazole and PO_2 histography, apparently because of tumor hypoxia heterogeneity[32-34]. Despite the actual relation with hypoxia remains difficult to evaluate, ^{18}F-FMISO has certainly shown its clinical value as hypoxia tracer. Increased ^{18}F-FMISO uptake has been associated with significant worse overall survival in HNSCC[35]. Furthermore, ^{18}F-FMISO was found effective in selecting advanced HNSCC patients who might benefit from treatment

with the hypoxic cytotoxin tirapazamine next to chemoradiation[31]. ^{18}F-FMISO was also successful in predicting radiotherapy outcome and suitable for following the radiation-induced reoxygenation of head and neck cancer during radiotherapy[11,36-39]. Although hypoxia imaging using ^{18}F-FMISO-PET seems to be feasible and has prognostic value, the largest disadvantages using this PET radiopharmaceutical is the relatively short half-life time of ^{18}F-FMISO, which hampers late imaging that could enhance good contrast between hypoxia and normal tissues[40]. Kumar et al. were the first who reported on the synthesis of ^{18}F-FAZA[41]. They showed that ^{18}F-FAZA was less lipophilic than ^{18}F-FMISO; therefore, it has higher perfusion and faster clearance from blood, resulting in a better hypoxia-background ratio. *In vitro* en *in vivo* xenograft studies confirmed the superiority of ^{18}F-FAZA in hypoxia specificity, higher tumor/background (T/B) ratios and ^{18}F-FAZA was found to be an independent negative factor for tumor progression and could predict the success of hypoxia-sensitizing treatment of tirapazamine and radiotherapy[42-45]. Nevertheless, ^{18}F-FAZA was associated with a significantly poorer prognosis in patients with head and neck cancer treated with (chemo)radiotherapy[46]. Despite the relatively low sample size and the diversity of the included tumors sites, our study further strengthens the idea that ^{18}F-FAZA-PET scan is a reliable method for hypoxia imaging with prognostic potential.

There was a lack of studies matching hypoxia in specific hypoxic subvolumes of whole tumor specimens using preoperative hypoxia imaging with representative markers of tumor hypoxia on selected areas in removed specimen. Whole specimen analysis is of great importance, because of the known heterogeneity of tumor hypoxia within the tumor mass. In chapter 3, we aimed to assess tumor hypoxic subvolumes in laryngeal cancer performing a preoperative ^{18}F-FAZA-PET/CT scan before total laryngectomy and analyzing commonly used exogenous and endogenous hypoxia markers (pimonidazole, HIF1α, CA-IX and GLUT-1) on selected areas of whole laryngectomy specimens to determine whether ^{18}F-FAZA-PET is suitable to define hypoxic subvolumes within the tumor. Inter- and intratumoral heterogeneity of tumor hypoxia was observed on ^{18}F-FAZA-PET scan. Nine of the 11 tumors were found to be hypoxic with ^{18}F-FAZA-PET imaging. Hypoxia could also be detected with pimonidazole, HIF1α, CA-IX and GLUT-1 expression in some tumors. However, there was no clear association between the ^{18}F-FAZA-PET uptake values and the quantitative expression of immunohistochemical hypoxia markers suggesting that ^{18}F-FAZA uptake may reflect tumor hypoxia, but not necessarily correlate with the spatial distribution of the extent of hypoxia. There can be

several explanations for this finding. First, tumor hypoxia is a dynamic process due to constantly changing tumor micro environment, such as differences in dynamic blood flow and chaotic blood supply[47]. Therefore, tumor cells that are hypoxic today may or may not be hypoxic at a subsequent time point. Secondly, the lack of a clear association between hypoxic areas detected by ^{18}F-FAZA-PET imaging and immunohistochemical staining with hypoxia markers can also be explained by acute blood perfusion changes during the surgery in comparison to the PET scan thus changing oxygenation grade[48]. Furthermore, theoretically, geometrical mismatch can also be responsible for the lack of correlation. Ideally, the whole specimen should have been co-registered by 3D matching together with the scan, like microscopic autoradiography or for instance in the study of Daisne et al.[49]. Last, the low specificity of endogenous markers to hypoxia may also be an explanation. For instance, HIF1α, CA-IX and GLUT-1 accumulation can be observed under other conditions than hypoxia, e.g. hypoglycemia and acidosis[50]. Many studies question the accuracy of hypoxia inducible genes for detecting hypoxia, since they poorly correlate with hypoxia as measured by pimonidazole[51,52]. Altogether, based on chapter 2, we concluded that ^{18}F-FAZA is insufficiently validated to be used in hypoxia guided radiotherapy dose escalation protocols. Further studies are required, like studies on radiotherapy dose planning which has already been performed with ^{18}F-FMISO-PET[10] to confirm ^{18}F-FAZA as a marker for the extent of hypoxia and its use in everyday clinical practice.

EGFR and PTEN: markers to predict local control after radiotherapy

Activation of the EGFR signaling pathway is known to lead to increased proliferation, metastasis, angiogenesis and decreased apoptosis in tumor cells[53,54]. Furthermore EGFR overexpression is associated with resistance of tumor cells against chemo- and radiotherapy and thereby with worse clinical outcome in many different tumor types[16,17,55,56]. One of the possible explanations for this association is the activation of EGFR and its downstream signaling pathway PI3K/PTEN/AKT after exposure of ionizing radiation. This pathway plays a crucial role in cell survival by inhibiting apoptosis and leads to accelerated repopulation of tumor cells[15,54]. This could explain why patients with locally advanced HNSCC and patients overexpressing EGFR are sensitive to accelerated radiotherapy compared to conventional radiotherapy[15,54,57,58]. In other clinical trials, hyperfractionated radiotherapy (delivery of more than one radiotherapy fraction per day) has reduced the local recurrence rate significantly in patients expressing high levels of EGFR[59].

Various studies suggested that the use of EGFR inhibitors improves the outcome of patients with locally advanced HNSCC, especially when they are associated with radiotherapy. For instance, in a phase III study, Bonner et al. showed improvement of treatment efficacy with the association of radiotherapy to cetuximab, a monoclonal antibody targeting EGFR[20]. Based on the results of that trial, since 2006, the U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) have approved cetuximab as adjuvant treatment to radiotherapy in patients with locally advanced HNSCC.

Unfortunately, in HNSCC, conflicting results have been published concerning EGFR expression levels and clinical outcome. One of the explanations is that the significance of EGFR has been investigated in heterogeneous populations treated with different treatment modalities. In chapter 4 of the present thesis, we intended to study an association between EGFR protein expression by immunohistochemistry and local recurrence in a homogenous group of early stage supraglottic LSCC patients treated with radiotherapy. No significant relationship was found. Therefore, the immunohistochemical expression status of EGFR in early stage supraglottic LSCC does not contribute to the selection of altered radiotherapy schedules or radiotherapy with adjuvant cetuximab. However, we have not studied response to cetuximab in this thesis, because such patients are not included in our study and EGFR-targeted therapy is not part of the standard treatment of early stage LSCC. Our results suggest that EGFR expression is not adequate to select patients who might be benefit from cetuximab. First, because of almost all patients showed high EGFR expression; secondly, because there was no association with local control. This might contribute to previous studies, which found that EGFR expression cannot accurately identify patients who will benefit from EGFR-targeted therapy[60]. Future studies need to focus on markers other than EGFR expression to improve the identification of HNSCC patients who benefit to EGFR-targeted therapies.

Another mechanism for PI3K/AKT pathway activation is the loss of PTEN. The role of PTEN in tumorigenesis as an antagonist in the PI3K/AKT pathway is a common mechanism in various malignancies[13]. PTEN inactivation could theoretically lead to resistance to EGFR inhibitors. Frattini et al. showed in a series of colorectal cancer patients that PTEN expression loss distinguished responders from non-responder patients treated with cetuximab[61]. Studies in prostate cancer cells showed a comparable relation and PTEN reintroduction restored the cell response to cetuximab[62]. Recently, also in HNSCC loss of PTEN expression was

associated with resistance to cetuximab[63]. These finding imply that loss of PTEN expression is a better predictive marker for EGFR-targeted therapy than EGFR expression. However, the role of PTEN expression in selecting HNSCC patients who might benefit from radiotherapy as well as from adjuvant cetuximab treatment in combination with radiotherapy is presently not known and has to be studied further.

Interestingly, not the loss of PTEN expression but a high PTEN expression has been associated with genome stability including DNA double strand break (DSB) repair by regulating the protein RAD51[63-65]. In a cohort of oral/oropharyngeal HNSCC patients treated with surgery and postoperative radiotherapy, our group reported a correlation between overexpression of PTEN and increased radioresistance determined by poor local control[19]. In chapter 4, we found the same association of high PTEN expression and deteriorated local control in a well-defined, homogeneous group of early stage supraglottic LSCC. Our data suggest that in HNSCC the high PTEN expression is a more common mechanism related to response on radiotherapy than PTEN loss. And the role of PTEN in RAD51-associated genomic instability might be a mechanism to regulate this response to radiotherapy. Although more studies are needed to understand how PTEN regulated genomic instability and radioresistance, our data show that high PTEN expression is a promising prognostic marker in HNSSC. In addition, in HNSCC with high expression, PTEN-inhibition as targeted-therapy in addition to radiotherapy is another area of attention for the future. Results of clinical trials with PTEN inhibitors are needed first since PTEN inhibitors (vanadium (VO-Ohpic), peroxovanadium (bpV) and phenanthrenedione-related (SF1670) compounds) are presently only investigated in preclinical studies[66].

The role of the DNA-damage response pathway including ATM, Chk2 and p53 in local control

Radiotherapy affects cell growth by inducing DNA damage, including DNA double-strand breaks (DSB) which lead to cell death by the activation of a complex DNA damage response (DDR) pathway which controls cell cycle checkpoints, DNA repair and apoptosis. Central in the DDR is the protein kinase Ataxia Telangiectasia Mutated protein (ATM). In the sixties of the last century it was already reported that Ataxia-telangiectasia patients, who frequently carry mutations in the ATM gene, have a predisposition to malignancy and are hypersensitive to irradiation[67,68]. In other malignancies the ATM protein is a key protein involved in DSBs caused by

radiation. Upon activation of ATM, a variety of targets including Chk2 and p53 are activated resulting in cell cycle checkpoint activation and DNA repair. In chapter 5, our results strongly suggest the involvement of phosphorylated ATM in response to radiation in early stage LSCC. This is in line with previous findings in advanced stage cervical cancer patients[22]. We showed that high pATM immunostaining was related to poor response to radiotherapy. Studies on the relation of ATM expression in response to radiotherapy in HNSCC are limited and results are rather controversial. Our study focused on the active (phosphorylated) isoform of ATM, whereas most other studies focused on expression of ATM regardless of phosphorylation state. Theoretically, a tumor with a majority of cells with high amounts of pATM, is more efficient in signaling DNA damage and subsequent repair. As a consequence, such a tumor has a better chance to survive after radiotherapy, which leads to poor response to radiotherapy. Based on these results, specific targeting of the ATM kinase activity could be an option for future therapy for LSCC in tumors high levels of pATM. Previous studies showed that inhibition of ATM by either RNA interference or targeted drug application results in increased sensitivity to radiotherapy in different malignancies[69-72]. Two ATM inhibitors (M3541 and AZD0156) are being tested in phase 1 trials, one combined with fractionated palliative radiotherapy in patients with solid tumors and the other as monotherapy and in combination with olaparib or 5-fluorouracil, folinic acid and irinotecan, in patients with advanced-stage solid cancers[73]. At present no FDA approved ATM inhibitor is available yet. Because LSCC with high pATM expression represent 27% of the patients in our cohort (chapter 5), pATM expression might be a prognostic marker as well as a new option for ATM-directed therapy in HNSCC. Future research is warranted to explore the role of pATM.

To our knowledge, there are no studies available exploring the immunohistochemical expression of pChk2 in laryngeal cancer, particularly not in relation with radioresponse. In chapter 5, we found no significant association between high pChk2 expression en local control, but pChk2 was expressed in only 2.5% in our cohort. And finally, also, no correlation was found between p53, one of the substrates for both pChk2 and pATM, expression (observed in 45% cases) and local control. This is good agreement with other findings regarding the correlation between immunohistochemical expression of p53 and clinical outcome after radiotherapy in LSCC[74]. Based on our results, the ATM/Chk2 pathway most likely contributes to radioresponse in laryngeal cancer without the contribution of p53.

The regulator of the methylation status of the tumor genome also influence response to therapy

During tumorigenesis, the expression of tumor suppressor genes and oncogenes can be altered by changes in promoter methylation. Increased methylation of the promoter-regions CpG islands of tumor suppressor genes and concomitant decreased methylation of the promotor-regions of specific proliferation-linked genes gradually increase tumor progression[25]. These changes are thought not only to be important in tumor progression but also in therapy response, invasion and metastasis[25,75-77]. The DNA methyltransferases (DNMT's) including DNMT1, DNMT3 and DNMT3b play an important role in the methylation process by adding methyl-groups to CpG dinucleotides and are involved in both de novo and maintenance of methylation status of the genome[78]. DNMT1 overexpression has been reported with aberrant DNA methylation in solid tumors resulting in poor prognosis of cancer patients[25,26]. DNMT1 expression has been reported as a potential prognostic marker in solid tumors, including HNSCC[79-81]. In chapter 6, we also found this relation of high DNMT1 expression and worse local control in our series of early stage LSCC treated with radiotherapy. Since high expression of DNMT1 was linked to poor local control, treatment options to inhibit DNMT1 becomes feasible especially as numerous inhibitors are available. DNMT1 inhibitors were already demonstrated to sensitize HNSCC cell lines to irradiation[82]. Epigenetic therapies, such as with DNMT inhibitors (azacitidine, decitabine, zebularine) lead to hypomethylation of DNA and represent new treatment options to modulate the radiation sensitivity of tumors. Azacitidine and decitabine are approved by the FDA and EMA for the treatment of acute myeloid leukemia, chronic myelomonocytic leukemia and myelodysplastic syndromes[83]. So far, no studies have been published which compare concomitant DNMT1 inhibitors and radiotherapy to radiotherapy alone in laryngeal carcinomas. This should be an area of future research. More interestingly in relation to low or high DNMT1 expression profiles.

In summary, this thesis presented insights in several mechanisms of response to radiotherapy in early stage laryngeal cancer. Our analysis revealed that several biological tumor markers involved in different pathways do not only have prognostic values for the response to treatment but might offer new opportunities for specific gene/pathway-targeted therapeutic intervention. Consequently, our findings might contribute to appropriate selection of patients who are likely to benefit from targeted therapies. Ultimately, this will support optimization of laryngeal cancer treatment and thereby improve local control and overall survival rates.

FUTURE PERSPECTIVES

Prognostic value of markers

In this thesis we did not find the perfect prognostic marker for early stage laryngeal carcinoma treated with radiotherapy which could select patients that will benefit from other treatment than current radiotherapy protocols like for instance radiotherapy dose escalation in specific tumor areas, primary surgery like partial laryngectomy or targeted therapy whether or not combined with radiotherapy. However, we gained valuable information which gives opportunities for future research. Since no association was found between hypoxia markers and ^{18}F-FAZA-PET, the spatial sensitivity of ^{18}F-FAZA for hypoxia is questionable. Yet, we found inter- and intratumoral differences in ^{18}F-FAZA uptake which still may have distinctive prognostic value. Future studies dealing with hypoxia imaging and specifically with ^{18}F-FAZA-PET have to focus on better spatial resolution. The traditional maximum standard unit value (SUV_{max}) or T/B ratio may not reflect the changes in global tumor microenvironment. They are characteristics of the single voxel with the least oxygenation status within the tumor. It is unlikely that single hypoxic voxel measurement within the tumor reflect the oxygenation status of the entire tumor volume. Hence, further studies should incorporate voxel-by-voxel analysis, which provides detailed information about the hypoxic distribution across the entire tumor rather than a single voxel. This will bring us more understanding of tumor biology and characterize the tumor heterogeneity.

The majority of studies reporting on prognostic markers in laryngeal cancer only investigated one or few related biological markers in small and often heterogeneous patient populations. As a result, often contradictory conclusions are drawn in different studies reporting on the same marker[74]. Although the promising prognostic value of PTEN, pATM and DNMT1 expression in early stage LSCC treated with radiotherapy has to be confirmed in larger independent cohorts, in this thesis we have shown that the clinical value for the individual patient of each of these individual markers is not sufficient for predicting response to radiotherapy. It is therefore expected that a marker panel consisting of various tumor markers as well as clinical markers, is a more feasible approach for clinical application and improvement of the clinical value to predict response to radiotherapy. For instance, an mRNA expression profile consisting of 696 genes was reported with a negative predictive value (NPV) of 89% for determining nodal spread in HNSCC[84]. Indeed this mRNA panel showed the highest NPV compared to most other available individual tumor markers to predict lymph node metastases in HNSCC[85,86], although the

NPV of the sentinel node biopsies procedure outperformed all these markers[87,88]. Using a combination of markers that represent specific pathway might be another approach to improve the predictive value. For example as reported in chapter 5, pATM (the central component of the DDR pathways controlling repair of DSB) is a promising prognostic marker for local control to radiotherapy. In this thesis the ATM-associated DDR pathway was studied by evaluating three of the many components studied in other malignancies frequently (ATM, Chk2, p53). These three components were selected based on a study that showed a better correlation between combined expression and patient survival compared to analysis of the separate compartments[89]. In our cohort of early stage LSCC only high pATM expression was prognostic, and both pChk2 and p53 expression did not affect this result (chapter 5). However, the DDR pathway is much more complicated and involves a large number of components (Figure 1) that each might further improve the prognostic value.

As other pathways have been associated with response to radiotherapy in HNSCC such as pathways involved in cell migration, invasion, inflammation and immunity, cell proliferation, cell cycle control, cell survival and hypoxia[74,90], multi-marker evaluation might increase the predictive value. Such approaches will also result in our understanding of the biology of resistance against radiotherapy in HNSSC and might enable us to select a small panel to use in clinical practice.

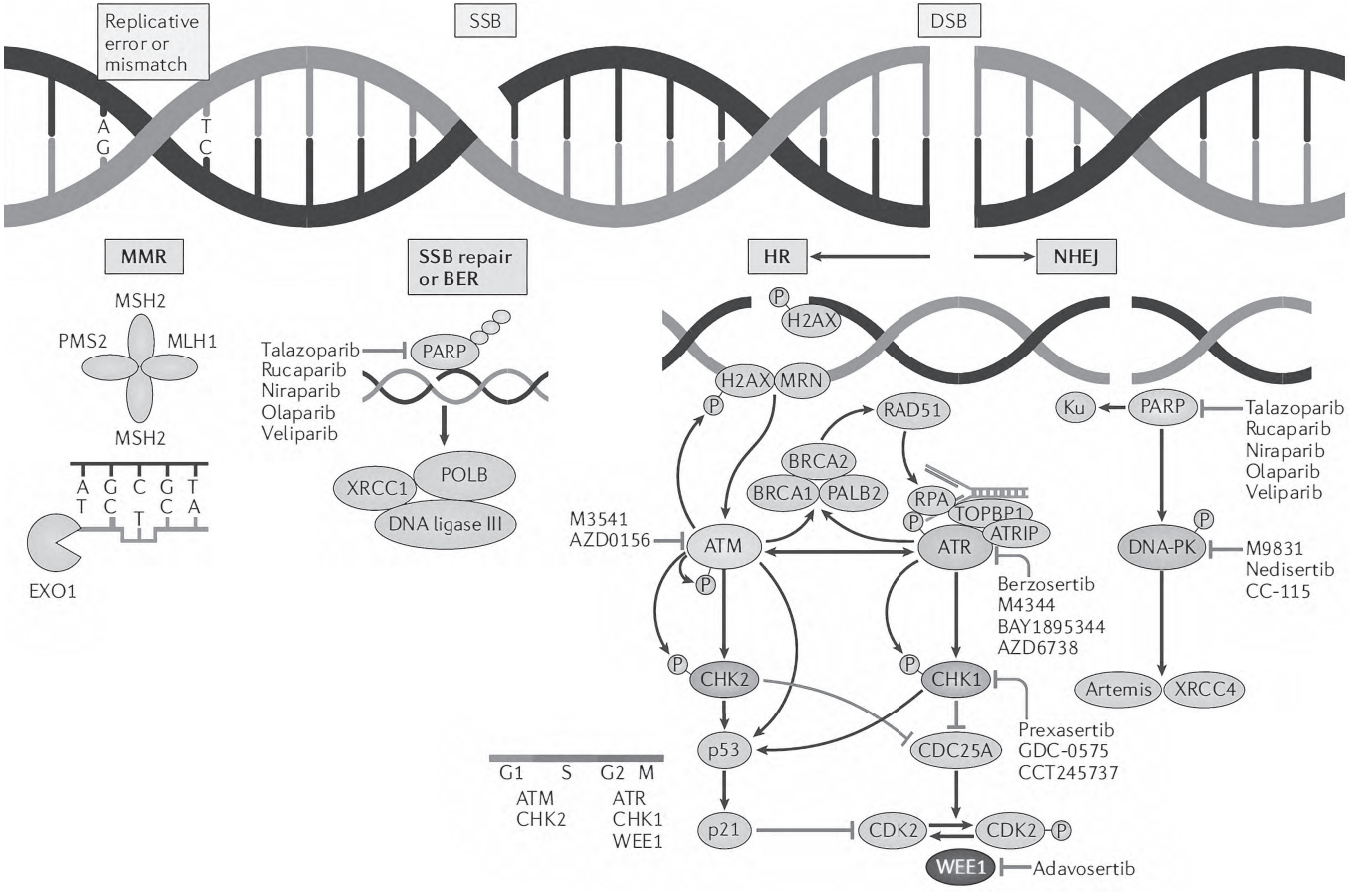
New innovative treatment modalities in HNSCC

Besides studying the prognostic value of the investigated tumor markers in this thesis, these same tumor markers could also act as possible targets for specific treatment modification in early stage LSCC.

Although the spatial sensitivity of ^{18}F-FAZA-PET for hypoxia remains questionable, future studies should certainly focus on the applicability of ^{18}F-FAZA-PET in radiation treatment, specifically targeted on hypoxia or hypoxic regions within the tumor. First, studies on ^{18}F-FAZA may focus on hypoxia imaging during the course of radiotherapy in HNSCC as hypoxic areas are to change during radiation. Later dose escalation trials based of ^{18}F-FAZA uptake may be introduced, like already performed with ^{18}F-FMISO[91,92].

Our findings are also promising, now therapies are becoming available that specifically target the prognostic markers that we found associated with resistance to radiotherapy in LSCC such as DNMT inhibitors (azacitidine and decitabine; FDA approved)[83] and ATM inhibitors (M3541 and AZD0156, now being tested in two phase 1 trials)[73]. Future clinical trials should include HNSCC.

Figure 1. DNA Damage Response Pathways. From *Pilié PG, Tang C, Mills GB, Yap TA. From State-of-the-art strategies for targeting the DNA damage response in cancer. Nat Rev Clin Oncol. 2019*[73], with permission.



The last years breakthrough of novel cancer treatments was dominated by antitumor immunotherapy[93]. The biological rationale for antitumor immunotherapy specifically in HNSCC is based on high tumor mutational load[94,95]. High tumor mutational burden was reported to be predictive for the efficacy of immune checkpoint inhibitors due to the occurrence of numerous mutations resulting in the expression of many neoantigenic proteins, which might serve as tumoral immune targets. Two PD-1/PD-L1 checkpoint inhibitors, nivolumab and pembrolizumab, are already approved by the FDA and EMA for use in advanced and recurrent stages of HNSCC[94,96,97]. The results from the KEYNOTE-048 study using immunotherapy in locally advanced or recurrent HNSCC are very promising[97]. This will also most probably result in new first line treatment modalities with either pembrolizumab alone or in combination with chemotherapy in recurrent or metastatic HNSCC. Although immunotherapy has shown to increase survival among patients with recurrent or metastatic HNSCC, concerns remain about selecting patients most likely to benefit from this treatment. Currently only PD-L1 checkpoint receptor expression using combined proportional score (cps), the number of PD-L1 positive cells (tumor, lymphocytes and macrophages) in relation to total tumor cells, is used as a predictive biomarker for response to immune checkpoint inhibitors in HNSCC based on the KEYNOTE-048 study by Burtness et al.[98].

In the Netherlands, the IMCISION study is currently studying hypoxia in relation to immunotherapy in HNSCC (ClinicalTrials.gov Identifier: NCT03003637). This is a phase IB/II trial to examine feasibility and safety of checkpoint blockade (nivolumab with or without ipilimumab) neoadjuvant to standard of care in advanced stage HNSCC. In addition, with this research protocol the potential impact of intratumoral hypoxia on tumor infiltrating lymphocyte (TIL) abundance, differentiation and effector function will be assessed, and the potentially divergent effects of T cell checkpoint blockade in areas of hypoxia and normoxia, through HX4-PET-guided tumor biopsies from hypoxic and normoxic tumor regions. It would be interesting to include the prognostic markers we have found in early stage LSCC in future studies, like the IMCISION. For instance, PTEN seems to be one these promising markers, as it has recently also been shown to play a pivotal role in antitumor immune regulation due to the activation of several escape mechanisms[99]. The tumor microenvironment has an almost full immunosuppressive profile by maintenance of genomic stability, cell survival/apoptosis, migration, and metabolism. Constitutive expression of PD-L1 by tumor cells seems to be driven by dysregulated signaling pathways, e.g. activation of the PI3K/AKT pathway[90]. It seems likely that the type of mechanism driving PD-L1 expression in tumor tissue can also affect its prognostic value.

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